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CD56$^{\text{bright}}$CD16$^+$ NK Cells: A Functional Intermediate Stage of NK Cell Differentiation

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Human NK cells comprise two main subsets, CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ cells, which differ in function, phenotype, and tissue localization. To further dissect the differentiation from CD56$^{\text{bright}}$ to CD56$^{\text{dim}}$ cells, we performed ex vivo and in vitro experiments demonstrating that the CD56$^{\text{bright}}$CD16$^+$ cells are an intermediate stage of NK cell maturation. We observed that the maximal frequency of the CD56$^{\text{bright}}$CD16$^+$ subset among NK cells, following unrelated cord blood transplantation, occurs later than that of the CD56$^{\text{bright}}$CD16$^-$ subset. We next performed an extensive phenotypic and functional analysis of CD56$^{\text{bright}}$CD16$^+$ cells in healthy donors, which displayed a phenotypic intermediary profile between CD56$^{\text{bright}}$CD16$^-$ and CD56$^{\text{dim}}$CD16$^+$ NK cells. We also demonstrated that CD56$^{\text{bright}}$CD16$^+$ NK cells were fully able to kill target cells, both by Ab-dependent cell cytotoxicity (ADCC) and direct lysis, as compared with CD56$^{\text{bright}}$CD16$^-$ cells. Importantly, in vitro differentiation experiments revealed that autologous T cells specifically encourage the differentiation from CD56$^{\text{bright}}$CD16$^-$ to CD56$^{\text{bright}}$CD16$^+$ cells. Finally, further investigations performed in elderly patients clearly showed that both CD56$^{\text{bright}}$CD16$^+$ and CD56$^{\text{dim}}$CD16$^+$ mature subsets were substantially increased in older individuals, whereas the CD56$^{\text{bright}}$CD16$^+$ precursor subset was decreased. Altogether, these data provide evidence that the CD56$^{\text{bright}}$CD16$^+$ NK cell subset is a functional intermediate between the CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ cells and is generated in the presence of autologous T CD3$^+$ cells.

The aim of the current study was to describe more precisely the CD56$^{\text{bright}}$CD16$^+$ NK subset and determine its role during NK cell differentiation. Our analysis of their function, phenotype, and frequencies during aging or after unrelated cord blood transplantation (UCBT), together with in vitro NK differentiation studies, strongly suggest that the CD56$^{\text{bright}}$CD16$^+$ NK cell subset is a functional intermediate between the CD56$^{\text{bright}}$ and CD56$^{\text{dim}}$ cells. Furthermore, we demonstrate that CD16 acquisition occurs in the presence of autologous T CD3$^+$ cells.

Materials and Methods

Patients and donors

Twenty-five patients (median age 43.5 y) underwent UCBT between 2005 and 2008 at either the Pitié-Salpêtrière or Hôtel Dieu hospitals (Paris, France) for high-risk hematopoietic malignancies. The cohort of these patients, mainly of Caucasian origin (24 out of 25), was previously described (18). Briefly, acute myeloid leukemia was the most common...
diagnosis (14 out of 25). All patients received a reduced-intensity conditioning regimen. 20 patients received the Minneapolis protocol containing cyclophosphamide 50 mg/kg at day 6, fludarabine 200 mg/m² for 5 d, and total body irradiation 2 Gy. For the last five patients, total body irradiation was replaced by 140 mg/m² melphalan. Cyclosporine and mycophenolate total body irradiation 2 Gy. For the last five patients, total body irradiation controls and cord blood samples were obtained, respectively, from the Etablissement Français du sang and the obstetrics department of the Pitié-Salpêtrière Hospital. All adult donors used for the phenotyping; functional and differentiation assays were aged between 18 and 60 y old. The rheumatology and rheumatology departments of Pitié-Salpétrière or Charles-Foix Hospitals furnished the samples from individuals aged ≥60 y or aged 17–60 y (mean age 68.8±7.2 y), and 26>80 y (mean age 87.1±4.9 y). All volunteers affirmatively stated, and their medical records confirmed, that they had no infectious, malignant, or autoimmune diseases during the 6 mo before the study and were without acute illnesses at the time of the sampling, but could present treatment for classical age-related pathologies as previously described (20). Patients and donors provided informed consent in compliance with the ethics committee guidelines before peripheral blood samples were collected for the study.

Flow cytometry
Phenotypes were realized on whole blood. Cells were stained using the appropriate Ab mixture: anti-CD3 (UCHT1; Beckman Coulter), anti-CD56 (N901; Beckman Coulter), anti-CD16 (3G8; Beckman Coulter; or VEP13; Miltenyi Biotec); anti-CD94 (HP9; Abcam), or anti-granzyme B (Gb11; Abcam). Degranulation, ADCC, and cytolytic assays were performed in 96-well U-bottom plates (BD Falcon). Depending on the assay, 10⁵ autologous CD3⁺ T cells were added to NK cell cultures. In some assays, CD3⁺ T cells were replaced by CD3⁺CD4⁺, CD3⁺CD8⁺, or CD3⁺CD56⁺ lymphocyte T subsets. When specified, T cells were physically separated from NK cells with transwells (eight-well strip insert; Nunc). Cytokines and culture media were renewed every 2 or 3 d. NK cells were characterized at indicated time points using the same panel of Abs as those used for the cell sorting.

Results
Kinetics of CD56brightCD16⁺ NK cell repopulation following UCBT
The phenotypic characterization of NK cells following UCBT revealed that the CD56brightCD16⁺ NK cell subset is highly expressed among whole NK cells following the transplantation. The CD56brightCD16⁺ subset increases very rapidly just after neutrophil engraftment, at 1mo post-UCBT, and then gradually decreases during the time examined (Fig. 1A). Interestingly, CD56brightCD16⁺ NK cells were present at 1mo posttransplantation, but increased to their maximum frequency 2mo after UCBT and remained stable during the following 12mo (Fig. 1B). These observations suggested that the CD56brightCD16⁺ cells are generated early posttransplantation and may be the precursors of the CD56brightCD16⁻ subset.

Phenotypic characterization of CD56brightCD16⁻, CD56brightCD16⁺, and CD56dimCD16⁺ NK cells in healthy individuals
To precisely determine the role of the CD56brightCD16⁺ NK cells, we performed an extensive phenotypic comparison with CD56bright CD16⁻ and CD56dimCD16⁺ NK cells in healthy donors. We firstly observed that CD56brightCD16⁻ and CD56brightCD16⁺ NK cells expressed numerous receptors in common with CD56dimCD16⁺ cells, such as Nkp30, Nkp46, DNAM-1, 2B4, LIR-1, and NKG2D (data not shown). However, some markers discriminated CD56bright NK cells, regardless of CD16 expression, such as CD94, NKG2A, CD127, CD27, CD62L, ITL-2, granzyme B, lymphocyte T subsets and perforin (Supplemental Fig. 1). More importantly, Fig. 2 shows that CD117 and CD25 were highly expressed in CD56brightCD16⁺ NK cells, and their expression progressively decreased in CD56brightCD16⁻ and CD56dimCD16⁺ NK cells. Conversely, the expression of CD8, granzyme A, CX3CR1, and pan-KIR progressively accumulated when looking sequentially at the NK cell subsets from CD56bright to CD56dim cells. Of note, high expression of KIR in the CD56brightCD16⁻ subset compared with the CD56brightCD16⁺ subset was also demonstrated individually for each KIR tested: KIR2DL1/DS1, KIR2DL2/3/4/5/6, and KIR3D1L (Supplemental Fig. 2). Together, these data suggest that CD56brightCD16⁺ cells may be an intermediate between CD56bright CD16⁻ and CD56dimCD16⁺ NK cells.
CD56<sup>bright</sup>CD16<sup>-</sup> and CD56<sup>bright</sup>CD16<sup+</sup> among NK cells after UCBT. Box and whiskers plots of CD56<sup>bright</sup>CD16<sup+</sup> (A) or CD56<sup>bright</sup>CD16<sup+</sup> (B) frequencies among NK cells 1 (M1), 2 (M2), 3 (M3), 6 (M6), and 12 (M12) mo after UCBT compared with healthy donors (Ctl) and cord blood samples (CB). C. Representative dot plots gated on CD3<sup+</sup>CD56<sup-</sup> NK cells. **p < 0.01, ***p < 0.001.

**CD56<sup>bright</sup>CD16<sup+</sup> NK cells are fully functional for cytolytic function**

To characterize functional properties of NK cells, regardless of CD16 expression, preliminary experiments were performed to determine appropriate experimental conditions. Indeed, CD16 is quickly downmodulated after encounter with K562 or RAJI target cells, which renders discrimination of the different subsets after degranulation assays impossible (Supplemental Fig. 3) (21, 22). Furthermore, the anti-CD16 3G8 clone is a blocking Ab of ADCC, which does not allow cell sorting for [51Cr] release assays (data not shown). For these reasons, we performed all experiments in the presence of the anti-CD16 VEP13 clone, which does not block ADCC. Fig. 3A shows that in the presence of the anti-CD16 mAb VEP13 clone, the expression of CD16 on NK cells is preserved after encounter of target cells, which allows analysis of NK subsets after degranulation assays (Fig. 3A).

We next compared the cytolytic capacities of CD56<sup>bright</sup>CD16<sup+</sup>, CD56<sup>bright</sup>CD16<sup+</sup>, and CD56<sup>dim</sup>CD16<sup+</sup> NK cell subsets in the presence of VEP13 anti-CD16 mAb. Fig. 3B shows that the CD56<sup>bright</sup> NK cells, expressing or not CD16, displayed similar degranulation ability against K562 target cells (p < 0.001 in both cases). However, chromium release assays revealed that CD56<sup>bright</sup>CD16<sup+</sup> cells were significantly less cytotoxic than CD56<sup>bright</sup>CD16<sup+</sup> (p < 0.01) and CD56<sup>dim</sup>CD16<sup+</sup> (p < 0.05) NK cell subsets against K562 cells, whereas CD56<sup>bright</sup>CD16<sup+</sup> and CD56<sup>dim</sup>CD16<sup+</sup> cells had similar direct cytolytic function (Fig. 3C). The high lytic ability of CD56<sup>bright</sup>CD16<sup+</sup> cells was confirmed by ADCC assays against RAJI cells covered with anti-CD20 mAb (rituximab). Indeed, both in degranulation and chromium release assays, CD56<sup>bright</sup>CD16<sup+</sup> cells were as efficient as CD56<sup>dim</sup>CD16<sup+</sup> cells (Fig. 3E, 3F). In contrast, CD56<sup>bright</sup>CD16<sup+</sup> cells could neither degranulate nor kill RAJI target cells in the presence of anti-CD20 mAb, in accordance with their lack of CD16 expression. Additionally, we demonstrated after IL-12/IL-18 stimulation that both CD56<sup>bright</sup>CD16<sup+</sup> and CD56<sup>bright</sup>CD16<sup+</sup> NK cell subsets produce large and equivalent amounts of IFN-γ compared with the low production of the CD56<sup>dim</sup> subset (Supplemental Fig. 4). Together, these data show that CD56<sup>bright</sup>CD16<sup+</sup> NK cells contain more cytotoxic properties than CD56<sup>bright</sup>CD16<sup+</sup> cells but also maintain the full ability to produce IFN-γ after cytokines stimulation.

**CD3<sup+</sup> T cells drive CD16 acquisition and subsequent ADCC ability of CD56<sup>bright</sup>CD16<sup+</sup> NK cells**

We next performed in vitro differentiation assays to further characterize the CD56<sup>bright</sup>CD16<sup+</sup> to CD56<sup>bright</sup>CD16<sup+</sup> differentiation. These experiments were performed with highly purified CD56<sup>bright</sup>CD16<sup+</sup> cells. Fig. 4A (left panels) shows that IL-2 alone was not able to drive CD16 expression on CD56<sup>bright</sup>CD16<sup+</sup> NK cells after 7 or 14 d of culture. By contrast, in the presence of autologous purified CD3<sup+</sup> T cells, a significant fraction of CD56<sup>bright</sup>CD16<sup+</sup> cells acquired CD16 (Fig. 4A, middle panels). To determine the role of the cell–cell contact requirements, similar experiments were performed in transwell plates. As shown in Fig. 4A (right panels), in the absence of cellular contacts, the CD56<sup>bright</sup>CD16<sup+</sup> NK cells showed reduced CD16 acquisition. We next evaluated the efficacy of CD4<sup+</sup>, CD8<sup+</sup>, and CD3 CD56<sup>bright</sup>CD16<sup+</sup> T cell subsets to induce CD16 acquisition by CD56<sup>bright</sup>CD16<sup+</sup> NK cells. In the presence of all of these CD3<sup+</sup> T cell subsets, similar proportions of NK cells expressing CD16 were observed (Fig. 4B). Importantly, kinetic studies revealed that in the presence of CD3<sup+</sup> T cells, expression of CD16 increased until day 14, when it reached a maximum level of between 25 and 30% (Fig. 4C). In an attempt to increase CD16<sup+</sup> expression on NK cells, we next performed experiments in the presence of autologous dendritic cells (DC). Immature DCs (iDC) were derived from purified CD14 monocytes in the presence of IL-4 and GM-CSF, whereas mature DCs (mDC) were obtained from iDC pulsed with LPS or bacillus Calmette–Guerin. Both iDC and mDC were cultured with or without CD3<sup+</sup> T cells to induce CD16 expression on CD56<sup>bright</sup>CD16<sup+</sup> cells. Unfortunately, the presence of iDC or mDC had no effect on CD16 expression (data not shown).

To determine the differentiation state of CD16<sup+</sup> cells generated in vitro in the presence of CD3<sup+</sup> T cells, we studied the expression of major differentiation markers including KIR, NKGD2A, ILT-2, and CD62L (Fig. 4D). We demonstrated that CD16<sup+</sup> NK cells expressed a significantly higher level of KIR than the CD16<sup+</sup> subset at all culture times examined, although this level remains low (~10%). Concomitantly, NKGD2A remained highly expressed on both subsets, whereas CD62L and ILT-2 decreased and increased, respectively, during the culture, independently of CD16 expression.

FIGURE 1. Frequency of CD56<sup>bright</sup>CD16<sup+</sup> and CD56<sup>bright</sup>CD16<sup+</sup> among NK cells after UCBT. Box and whiskers plots of CD56<sup>bright</sup>CD16<sup+</sup> (A) or CD56<sup>bright</sup>CD16<sup+</sup> (B) frequencies among NK cells 1 (M1), 2 (M2), 3 (M3), 6 (M6), and 12 (M12) mo after UCBT compared with healthy donors (Ctl) and cord blood samples (CB). C. Representative dot plots gated on CD3<sup+</sup>CD56<sup-</sup> NK cells. **p < 0.01, ***p < 0.001.
We further assessed cytolytic function acquisition by CD56\textsuperscript{bright}CD16\textsuperscript{−} NK cells after in vitro coculture with T cells. We tested direct cytotoxicity and ADCC ability of CD56\textsuperscript{bright} NK cell subsets after 28 d of culture. Fig. 4E shows that both NK cell subsets generated in vitro were able to degranulate against K562 target cells. More interestingly, degranulation against RAJI target cells covered with anti-CD20 mAb was restricted to the CD16-expressing subset.

These data suggest that T cells drive CD16 expression on CD56\textsuperscript{bright}CD16\textsuperscript{−} NK cells and their subsequent ability to perform ADCC.

*In vitro differentiation of CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells*

Next, a similar strategy was used to determine in vitro differentiation of CD56\textsuperscript{bright}CD16\textsuperscript{+} NK cells in the presence of CD3\textsuperscript{+} T cells. Fig. 5A shows that CD16 expression disappeared on half of the cells during the first days of culture and thereafter prog-
higher KIR expression was observed in in vitro-cultured CD56<sup>bright</sup>CD16<sup>+</sup> NK cells compared with CD56<sup>bright</sup>CD16<sup>-</sup> NK cells (Fig. 5B).

Aging is associated with the accumulation of CD16<sup>+</sup> NK cells among the CD56<sup>bright</sup> subset

Finally, we performed an analysis of CD16 expression among NK cells during aging. It is already known that the aging process is associated with fewer CD56<sup>bright</sup> NK cells and an accumulation of CD56<sup>dim</sup> NK cells in peripheral blood (20, 23). We showed that the percentage of CD56<sup>bright</sup> NK cells expressing CD16 increases linearly with aging (Fig. 6A). Indeed, when comparing people’s aging with <60 and >80 y old, we observed that older persons expressed 2–3-fold more CD16 in their CD56<sup>bright</sup> compartment (p < 0.001). Thus, younger donors expressed CD16 on ∼25% of CD56<sup>bright</sup> NK cells, whereas elderly people >80 y old expressed this marker on ∼50–75% of these cells. Fig. 6B and 6C show that this change is principally due to a decrease of the CD56<sup>bright</sup>CD16<sup>+</sup> (p < 0.01) and an increase of CD56<sup>bright</sup>CD16<sup>-</sup> cell counts (p < 0.01). These data clearly show that old age is associated with fewer CD56<sup>bright</sup>CD16<sup>+</sup> cells and an accumulation of both CD56<sup>bright</sup>CD16<sup>+</sup> and CD56<sup>dim</sup>CD16<sup>+</sup> NK cells in the periphery.

Discussion

The present study presents evidence that CD56<sup>bright</sup>CD16<sup>+</sup> NK cells are a functional differentiation intermediate between CD56<sup>bright</sup> and CD56<sup>dim</sup> cells. A recent paper of Dulphy et al. (15) showed that 3 mo after matched HSCT, the frequency of CD56<sup>bright</sup>CD16<sup>+</sup> NK cells was largely increased among NK cells. In this study, we confirm this observation and additionally show that the maximum level of CD56<sup>bright</sup>CD16<sup>+</sup> into NK cells is observed later than that of CD56<sup>bright</sup>CD16<sup>-</sup>. This suggests that the CD56<sup>bright</sup>CD16<sup>+</sup> cells may be an intermediate between CD56<sup>bright</sup>CD16<sup>-</sup> and CD56<sup>dim</sup> CD16<sup>+</sup> NK cell subsets. Concomitantly, in elderly individuals, more CD56<sup>dim</sup>CD16<sup>+</sup> cells associated with aging were previously reported (20, 23, 24). More importantly, in this study, we have determined the percentage of CD56<sup>bright</sup> cells expressing CD16 and observed that it is inversely associated with aging. These data suggest that old age favors an accumulation of more mature NK cell subsets, such as CD56<sup>bright</sup>CD16<sup>+</sup> and CD56<sup>dim</sup>CD16<sup>-</sup> cells, in detriment to the CD56<sup>bright</sup>CD16<sup>-</sup> precursor cells, as previously described for T and B cells (25). It was observed in older individuals both increasing of soluble IL-2R, which downregulated IL-2 activity (26), and IL-12p40 homodimers, which could act as an IL-12 antagonist (27). This could suggest that the disturbance of specific cytokine signaling could partially block the final maturation of NK cells at a stage of CD56<sup>bright</sup>CD16<sup>+</sup> NK cells, which are accumulated in older subjects.

To minimize this effect due to particular clinical situations, the main experiments of this study were performed in healthy donors. The CD56<sup>bright</sup>CD16<sup>+</sup> NK cell subpopulation represents 1.7 ± 1.6% of NK cells. An extensive phenotypic analysis revealed few differences between CD56<sup>bright</sup>CD16<sup>-</sup> and CD56<sup>bright</sup>CD16<sup>+</sup> cells. CD56<sup>bright</sup>CD16<sup>-</sup> NK cells showed intermediate expression levels of CD25, CD117, CD8, CX3CR1, KIRs, and granzyme A between the CD56<sup>bright</sup>CD16<sup>-</sup> and the CD56<sup>dim</sup>CD16<sup>+</sup> cells. This was in accordance with Caligiuri’s model (28), which predicts CD117 loss and KIR acquisition during stage 4 (CD56<sup>bright</sup>) to stage 5 (CD56<sup>dim</sup>) transition. Specific cytokine molecules also revealed the maturity of CD56<sup>bright</sup>CD16<sup>+</sup> NK cells. Indeed, the CD56<sup>bright</sup>CD16<sup>-</sup> NK cells expressed almost exclusively granzyme K, whereas CD56<sup>bright</sup>CD16<sup>+</sup> additionally overexpressed granzyme A. Finally, CD56<sup>dim</sup>CD16<sup>+</sup> NK cells lost granzyme K.
and expressed abundant levels of granzyme A, granzyme B, and perforin. This succession of granzymes in NK cell subsets is similar to what is observed during memory CD8+ T cells differentiation (29).

Importantly, we show that CD56 bright CD16+ and CD56 dim CD16+ cells have similar cytotoxic functions, greater than those of CD56brightCD16− precursor cells. Furthermore, the progressive increase of CX3CR1 expression on CD56 bright CD16+, CD56 bright CD16+, and CD56 dim CD16− NK cell subsets suggests an evolution in the acquisition of migratory properties to inflammations sites (30). Remarkably, we also found that both CD56 bright CD16− and CD56 bright CD16+ cells displayed equivalent high abilities to
produce IFN-γ after an IL-12/IL-18 stimulation compared with CD56dimCD16+ cells. Altogether, these data demonstrate that CD56brightCD16+ NK cells share functional properties of the CD56brightCD16− and CD56dimCD16− subsets and suggest that CD16 acquisition could be used as a measure of NK cells' functional maturity.

These ex vivo data were confirmed by in vitro NK cell differentiation experiments. Our data show that culture of CD56bright

![FIGURE 5. Modulation of NK receptor on CD56brightCD16− or CD56brightCD16+ NK cells cultured in the presence of autologous T cells. A. Box and whiskers plots of 12 different cultures (left panel) and representative pictures (right panel) of CD16 expression on CD56brightCD16− NK cells after 0, 7 (d7), 14 (d14), and 28 d (d28) of cocultures with CD3+ T cells. Analysis are based on CD3+CD56− gated NK cells. B. Evolution of differentiation markers on CD56brightCD16− or CD56brightCD16+ NK cells after 7 (d7), 14 (d14), and 28 d (d28) of coculture of with CD3+ T cells. Data are represented as mean and SD of three independent cultures. *p < 0.05, **p < 0.01, ***p < 0.001.](http://www.jimmunol.org/)

![FIGURE 6. Old age is associated with a decrease of CD56brightCD16− cells and an accumulation of CD56brightCD16− and CD56dimCD16+ NK cell subsets. A. Expression of CD16 on CD56bright NK cell subsets from donors <60 (n = 29; 18 < x < 60) and between 60 and 80 (n = 22; 60 < x < 80), or >80 (n = 17; x > 80) y old. B. Representative patterns of two independent donors gated on CD3+CD56+ NK cells. C. Absolute values of CD56brightCD16−, CD56brightCD16+, and CD56dimCD16+ NK cell subsets from donors ranged between 18 and 60 (n = 20; 18 < x < 60), between 60 and 80 (n = 22; 60 < x < 80), or >80 (n = 17; x > 80) y old. *p < 0.05, **p < 0.01, ***p < 0.001.](http://www.jimmunol.org/)
CD16− cells with IL-2 alone is not sufficient to induce CD16 expression, as previously reported (19). This contrasts with results reported by two other groups, describing an overexpression of CD16 after culture with IL-2 alone (4, 8). This discrepancy could be explained by cell-sorting conditions, as these groups sorted all CD56bright NK cells, regardless of CD16 expression. However, our results are in line with the findings of in vitro NK cell differentiation from CD34+ stem cells (31). Freud et al. (31) showed that in medium containing IL-2 or IL-15, it was only possible to generate a CD56bright-like subset, expressing no CD16 and KIR, but high amounts of CD117.

More importantly, we provide solid evidence that CD3+ T cells are a key component to drive acquisition of functional CD16 during the CD56brightCD16− to CD56brightCD16+ transition. These data are in line with Fehniger et al. (32), who showed that CD56bright NK cells were found in the parafollicular T area of lymph nodes in direct contact with CD3+ T cells. Additionally, Freud et al. (31) demonstrated that autologous activated T cells were able to induce the differentiation of CD34+CD45RA+ integrin β7+ stem cells into CD56bright NK cells. More recently, we have shown that after haploidential HSCT, NK cell reconstitution was positively influenced by the amount of T cells contained in the graft (33). This work also showed that NK–T cell interactions seemed to drive CD16 acquisition, although a slight containment in the graft (33). This work also showed that NK–T cell interactions seemed to drive CD16 acquisition, although a slight containment in the graft (33).

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Disclosures

The authors have no financial conflicts of interest.

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